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Supplementary Materials for

cFLIP$_L$ protects macrophages from LPS-induced pyroptosis via inhibition of complex II formation

Hayley I. Muendlein, David Jetton, Wilson M. Connolly, Keith P. Eidell, Zoie Magri, Irina Smirnova, Alexander Poltorak*

*Corresponding author. Email: alexander.poltorak@tufts.edu

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This PDF file includes:

Materials and Methods
Figs. S1 to S4

Other Supplementary Material for this manuscript includes the following:
(available at science.sciencemag.org/content/367/6484/1379/suppl/DC1)

MDAR Reproducibility Checklist (.pdf)

Materials and Methods

Mice and Macrophages

C57BL/6 (B6), *Casp1*$^{-/-}$*Casp11*$^{-/-}$, *Ticam1*$^{-/-}$ *(Trif*$^{-/-}$*)* and *Nlrp3*$^{-/-}$ mice were obtained from The Jackson Laboratory. Mice were housed according to protocols approved by the Tufts University Medical School Animal Care and Use Committees. Femurs from *Gsdmd*$^{-/-}$ mice were donated by Dr. K. Fitzgerald, and were generated by Dr. V. Dixit. Femurs from *Asc*$^{-/-}$ mice and immortalized BMDMs expressing fluorescently tagged ASC were generously provided by Dr. K. Fitzgerald. Ripk1$^{K45A/K45A}$ (RIP1Ki) mice were provided by Dr. A. Degterev. Femurs from *Rip3Casp8*$^{-/-}$ mice were donated by Dr. K. Fitzgerald, and were originally generated by Dr. D. Green. Bone marrow was isolated from the long bones of mice, propagated in RPMI containing 20% FBS, 2% Pen-Strep and 30% L cell supernatant on non-tissue culture treated Petri dishes. Once differentiated, bone marrow derived macrophages (BMDMs) were plated for experiments at a density of 1 x 10^6 cm^2 in RPMI containing 20% FBS and 2% Pen-Strep. *Yersinia* based experiments were conducted in the absence of antibiotics.

Reagents

Lipopolysaccharide (LPS) *S. minnesota* R5 (10 ng/ml unless otherwise indicated), Lipoteichoic acid (LTA) *S. aureus* (2 µg/ml), polyinosinic:polycytidylic acid (poly(I:C), 20 µg/ml)and 5z7 (125 nM) were purchased from Sigma. Nigericin (1 µM) was purchased from InvivoGen. Caspase-3/7 Inhibitor I was purchased from Cayman Chemical. RNase-free KCl (2 M) was purchased from ThermoFisher Scientific. Propidium iodide (10 µg/ml) was purchased from Life Technologies. Annexin V, Alexa Fluor 350 conjugate (A23202) was purchased from Invitrogen.

Yersinia Growth Conditions

Wild-type and ΔyopJ IP2666 *Y. pseudotuberculosis* bacterial strains were generously provided by Dr. Ralph Isberg. Bacteria were grown from frozen glycerol stocks on LB plates containing Irgasan (Sigma). Cultures were grown overnight at 26°C in 2XYT broth, diluted to an OD_{600} of 0.2 and grown at 26°C for 2 additional hours prior to a shift to 37°C for 2 hours. Macrophages were infected at multiplicity of infection (MOI) of 30 CFU/cell.

Kinetic Microscopy

The Cytation3 automated microscope was used for kinetic macrophage imaging assays, and built-in environmental control maintained 37°C, 5% CO_2 for the duration of the assay. Macrophages were seeded at a density of 1 x10^6 cm^2 in RPMI on 1.17 mm thick glass bottom imaging plates. To generate kinetic cell death curves, cells were imaged at 30-min intervals at 4X magnification to capture approximately 5,000 cells/field of view. Propidium iodide (PI) incorporation was detected at 617 nm, and PI^+ nuclei were counted. Wells treated with 0.1% Triton X-100 were used as controls for 100% cell death. For Annexin V/ PI stained 20X images as in 2B, macrophages were seeded in RPMI buffered at pH 7.2-7.5 (1 mM HEPES) and supplemented with 2 mM $CaCl_2$ to allow for Annexin V binding. Cells were imaged at 30-min intervals at 20X magnification, PI incorporation was detected as at 4X, and AnnexinV was detected at 350 nm.

ELISA

Two, four or six-hours after indicated treatments, cell supernatants were collected and cytokine secretion was measured by ELISA. Murine IL-1β DuoSet ELISA Kits (DY401) were used according to the manufacturer's instructions.

Immunoblotting

After indicated treatments, cell supernatants were collected and cells were lysed in 1X Laemmli Buffer containing 5% β-mercaptoethanol, boiled for 15 min and incubated on ice for 15 min. For indicated conditions, proteins were precipitated from cell supernatant by methanol-chloroform extraction, and precipitated proteins were resuspended in 1X Laemmli Buffer containing 5% β-mercaptoethanol and processed as above prior to loading and running on SDS-PAGE gels. Primary antibodies against IL-1β (12242), cFLIP (56343), Casp8 (8592), Casp3 (9665), Casp7 (9492), Casp9 (9508), Casp11 (14340), Casp1 (24232), RIP1 (3493), pRIP1 (Ser166) (31122) and GAPDH (2118) were purchased from Cell Signaling Technologies. GSDMD antibody (ab209845) was purchased from Abcam, and FADD antibody (05-486) was purchased from Millipore Sigma. Secondary antibodies, anti-rabbit IgG (H+L) (DyLight™ 800 4X PEG Conjugate) (5151), anti-mouse IgG (H+L) (DyLight™ 800 4X PEG Conjugate) (5257) and HRP-linked anti-rat IgG antibody (7077) were purchased from Cell Signaling Technologies.

RNA Preparation and Analysis

Total RNA was isolated from 5 x 10^5 cells after indicated treatments using TRIzol (Invitrogen), following the manufacturer's instructions. cDNA was synthesized by reverse transcription, performed using M-MuLV reverse transcriptase, RNase inhibitor, random primers , and dNTP mix (New England BioLabs). cDNA was analyzed for relative mRNA levels using SYBR Green (Applied Biosystems), and gene specific primers. Gapdh was used to normalize mRNA levels, and post amplification melting curve analysis was performed to confirm primer specificity.

Quantitative PCR Primers:

Cflar: (F) 5'-CCGTGAAGAGACTTACAGGATG-3',
(R) 5'-GATATGATAGCCCAGGGAAGTG-3'.
Gapdh: (F) 5'-GGAGAGTGTTTCCTCGTCCC-3',
(R) 5'-TTCCCATTCTCGGCCTTGAC-3'.

Next Generation RNA sequencing

Total RNA was isolated from unstimulated, LPS-stimulated, and LPS/5z7 stimulated B6 BMDMs using TRIzol. A TrueSeq kit was used to make a directional cDNA library. Seventy-five bp reads from cDNA libraries were generated on HiSeq (Illumina) and aligned using TopHat2 and Cufflinks software. Log-transformed values of genes that were upregulated after LPS stimulation over unstimulated(>3 fold), and downregulated after LPS/5z7 compared to LPS were displayed by heat map. Genes were clustered by GeneOntology (GO) Molecular Function.

Lentivirus constructs and transduction

Target hairpin sequences were identified using the Genetic Perturbation Platform from the Broad Institute. Selected sequences for $cFLIP_R$ target a sequence present in the 3'UTR (5'-CCAGTCTCAGAGATGTAATAA - 3') and for $cFLIP_L$ target a sequence in exon 8 (5'-TTCCCAAGTCACATGACATAA - 3'). Oligos were ordered from IDT and target sequences were ligated into the pLKO.1 targeting vector (plasmid 10879; Addgene, Cambridge, MA).

Scramble control vector was purchased from Addgene (plasmid 1864). For $cFLIP_L$ add back and overexpression experiments, mutagenized $cFLIP_L$ sequences were ligated into pLEX lentiviral vector. Lentiviral particles were generated in the 293T cell line by transfection with packaging vector psPAX2 (plasmid 12260; Addgene) and the VSV-G pseudotyping vector pMD2.G (plasmid 12259; Addgene). Resultant lentiviral particles were used to transduce BMDMs on day 4 of the 7-day differentiation period, followed by puromycin selection (48 hours).

siRNA based $cFLIP_L$ Knockdown

DsiRNA duplexes were generated by IDT to target $cFLIP_L$ (5'-AUUUGUGGAAUACCGUGACAGUCAA-3', 3'-UCUAAACACCUUAUGGCACUGUCAGUU-5'). Scrambled Negative Control DsiRNA (Cat # 51-01-19-09) was purchased from IDT. DsiRNA were reconstituted to 100 uM, and nucleofection of indicated BMDMs was performed using the Lonza Amaxa Mouse Macrophages Nucleofector Kit, according to manufacturer instructions. After electroporation, cells were allowed to rest for 12 hours prior to stimulation for necessary experiements.

ASC Speck Quantification and High Magnification Imaging

To quantify ASC^+ cells and perform high magnification imaging of ASC specks, macrophages were seeded at a density of 1×10^6 cm^2 in RPMI on 1.17-mm-thick glass bottom imaging plates. For LPS/Nigericin conditions, cells were stimulated with LPS (100 ng/ml) for 2 hours prior to stimulation with Nigericin (1µM) for 1 hour. For LPS conditions, macrophages were stimulated with LPS (10ng/ml) for 2 hours. Cells were fixed in 4% paraformaldehyde for 15 minutes, blocked in 1XPBS (5% FBS, 0.3% Triton X-100), and incubated overnight with anti-ASC antibody (Cell Signaling Technologies, 67824), followed by a 2-hour incubation in the presence of Alexa Fluor 488-conjugated-Goat-anti-Rabbit IgG (Invitrogen A-11034), and Hoechst 33342 fluorescent stain. Alternatively, to visualize ASC speck formation kinetically, ASC-GFP immortalized BMDMs were stimulated as described above in the presence of propidium iodide and Hoechst 33342 stain. To quantify the percentage of ASC^+ cells, the Cytation3 automated microscope was used to image cells at 4X magnification to capture approximately 5000 cells/field of view in quadruplicate. Hoechst stain was detected at 350 nm and Hoechst+ nuclei were counted to provide total cell count. The ASC signal was detected at 488 nm and ASC specks (1 um-3 um) were counted. To perform high-magnification kinetic imaging of ASC specks in LPS and LPS/Nigericin stimulated cells, macrophages were prepared as above and imaged at 60X on a BioTek Lionheart automated microscope.

FADD Immunoprecipitation

Cells were plated on 10 cm dishes, stimulated as indicated and harvested in immunoprecipitation lysis buffer (0.5% Triton X, 50 mM Tris Base (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1X protease inhibitor cocktail). Lysed cells were rotated for 60 min at 4 degrees C with intermittent vortexing, centrifuged at 5,000 X *g* for 5 min, and the supernatant was incubated with α-FADD antibody-conjugated Protein G agarose beads (Cell Signaling Technology 37478). Samples were washed three times in immunoprecipitation lysis buffer, and protein complexes were eluted with 1X Laemmli buffer containing 5% β-mercaptoethanol at 90 °C for 15 min.

Quantification and Statistical Analysis

Error bars in qPCR and ELISA experiments represent the standard deviation of three independent experiments. Data from kinetic cytotoxicity and ASC^+ cell quantification experiments are representative of three or more experiments, and error bars represent the standard deviation between duplicate or triplicate samples. Immunoblots are representative of three or more independent experiments. Significance was determined using a one-way or two-way ANOVA as appropriate: ns (non-significant) $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

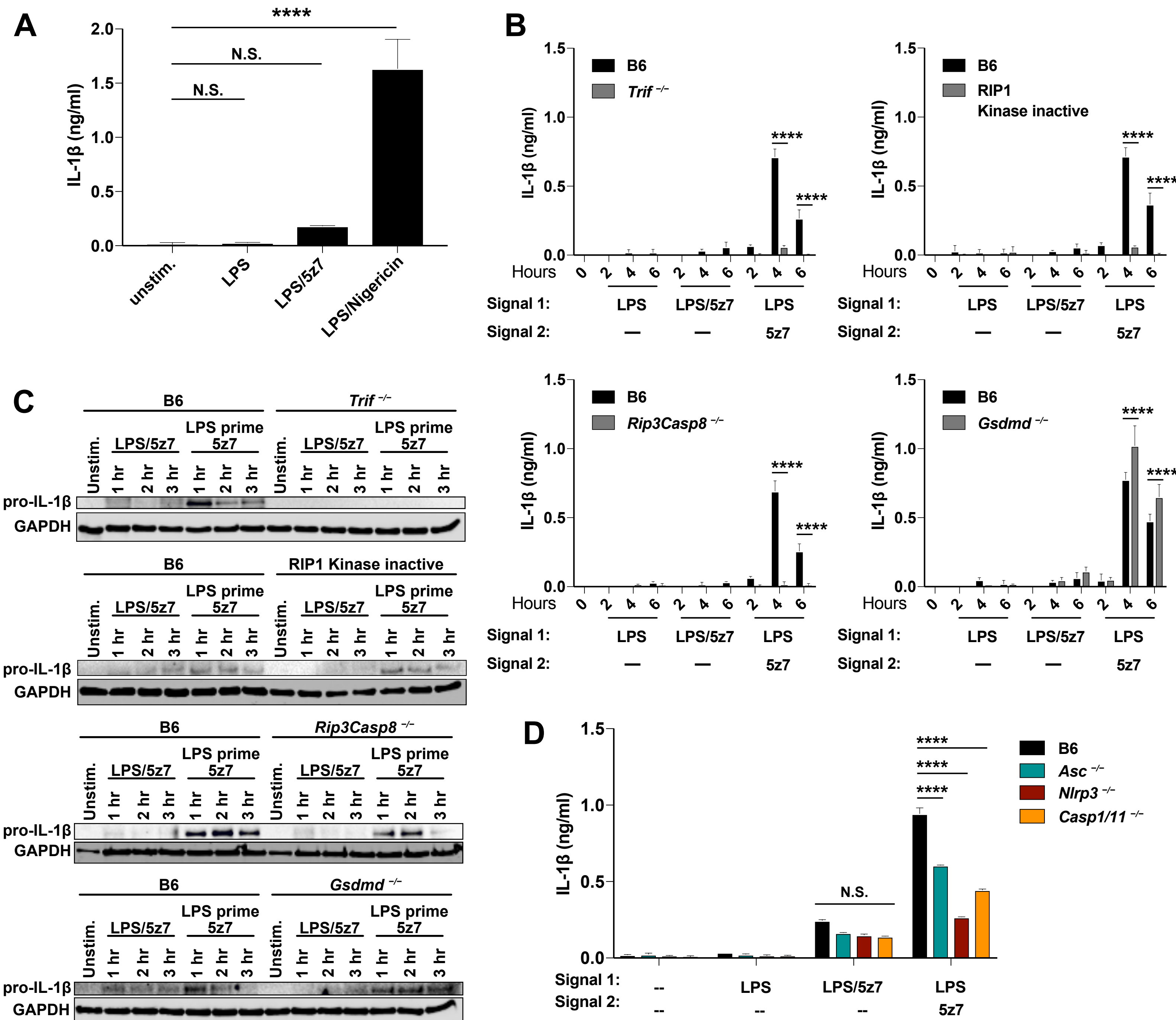

Fig. S1. (A) IL-1β release from B6 BMDMs 6 hours after indicated treatments. (B) IL-1β release from B6, RIP1 kinase inactive, TRIF, RIP3/CASP8 and GSDMD deficient BMDMs after 2, 4 or 6 hours of indicated treatments. (C) pro-IL-1β detected in the cell lysate of indicated BMDMs, stimulated concurrently with LPS/5z7 or LPS-pre-primed/5z7 for 1, 2 or 3 hours. (D) IL-1β release from indicated BMDMs after 6 hours of treatment. For all panels, LPS pre-priming (10 ng/ml or 100 ng/ml) occurred 4 hours prior to the addition of 5z7 or Nigericin respectively. Immunoblot data are representative of three or more independent experiments. IL-1β release data are presented as the mean ± SD for triplicate wells from three or more independent experiments. Analysis of covariance (ANOVA) was used for comparison between groups: ns, nonsignificant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

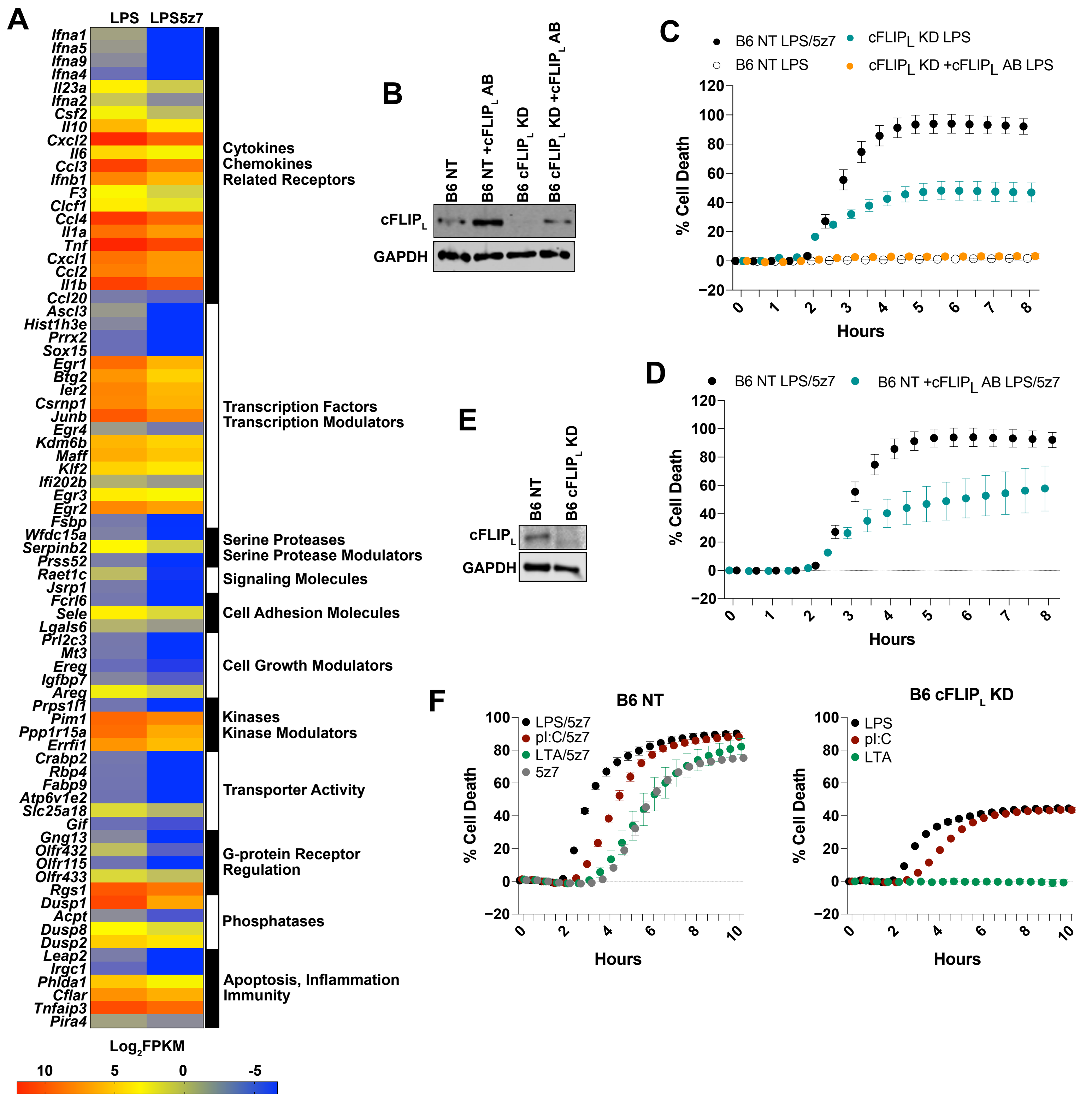

Fig. S2. (A) Genome-wide mRNA levels presented for genes upregulated after 1 hour of LPS stimulation compared to unstimulated, and downregulated after 1 hour of LPS/5z7 treatment compared to LPS in B6 macrophages. (B) $cFLIP_L$ protein levels in B6 BMDMs knocked down for $cFLIP_L$ or transduced with a non-targeting control (NT), ± reconstitution or overexpression of exogenous $cFLIP_L$ (Addback, AB). (C, D) Cell death in B6 NT control, B6 $cFLIP_L$ KD BMDMs ± reconstitution (C) or overexpression (D) of exogenous $cFLIP_L$ after stimulation with LPS or LPS/5z7 (extent of knockdown, reconstitution or overexpression of $cFLIP_L$ is shown in panel B). (E) $cFLIP_L$ protein levels in B6 BMDMs knocked down for $cFLIP_L$ or transduced with a non-targeting control (NT). (F) Cell death in B6 NT control BMDMs stimulated with various TLR agonists+5z7, and $cFLIP_L$ knocked down BMDMs stimulated with TLR agonists alone (extent of knockdown is shown in panel E). Data presented are representative of three or more independent experiments. Cell death data are presented as the mean ± SD of triplicate wells.

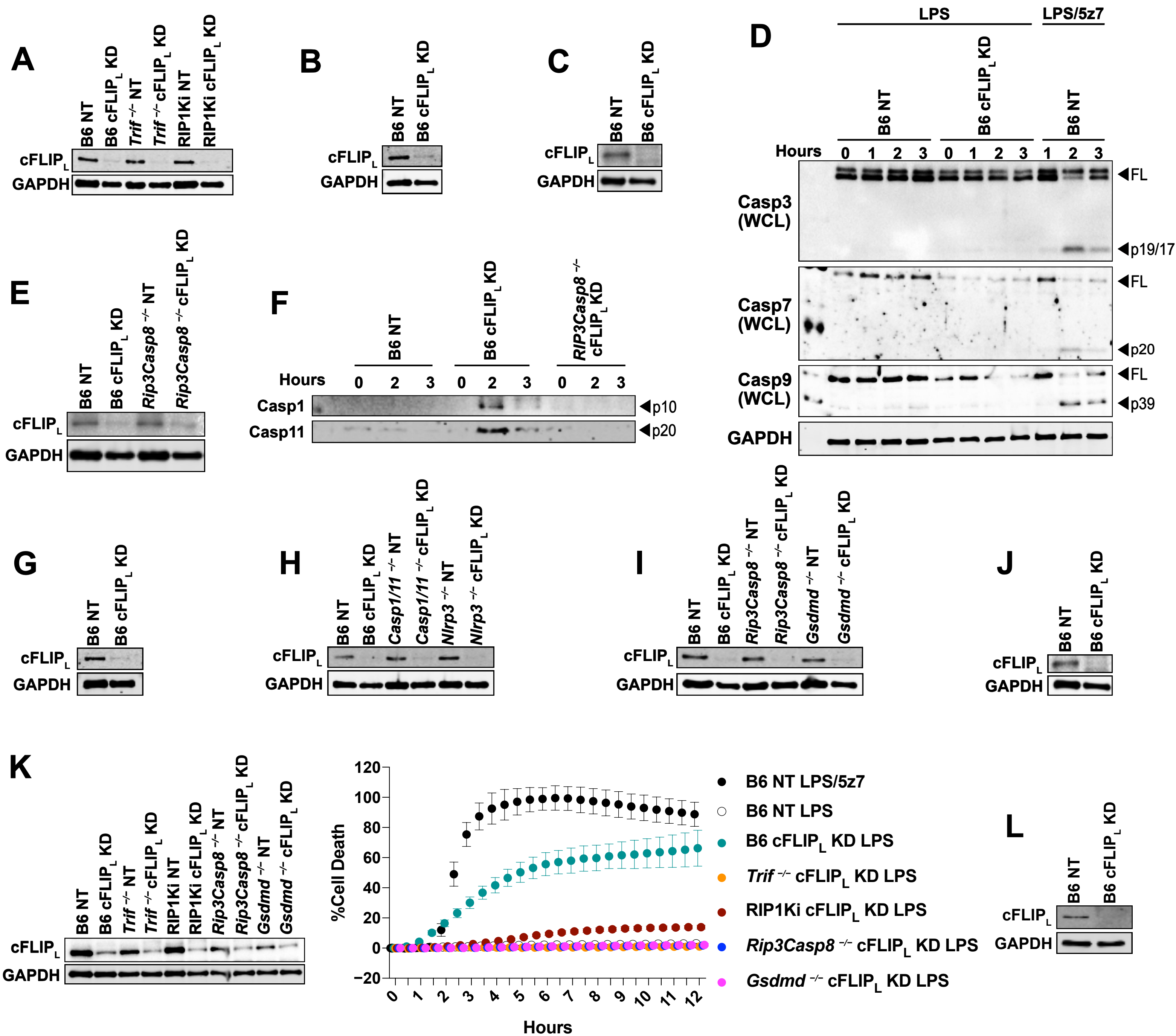

Fig. S3. (A-C) cFLIP$_L$ protein levels in indicated BMDMs knocked down for cFLIP$_L$ or transduced with a non-targeting control (NT). (D) Full length and cleaved caspase products from whole cell lysates of B6 NT control or cFLIP$_L$-KD BMDMs (extent of cFLIP$_L$ knockdown is shown in panel C). (E) cFLIP$_L$ protein levels in B6 and *Rip3Casp8*$^{-/-}$ BMDMs knocked down for cFLIP$_L$ or transduced with a non-targeting control (NT). (F) Cleaved CASP1 and CASP11 precipitated from the supernatant of B6 and *Rip3Casp8*$^{-/-}$ NT control or cFLIP$_L$ KD BMDMs (extent of cFLIP$_L$ knockdown is shown in panel E). (G-J) cFLIP$_L$ protein levels in indicated BMDMs knocked down for cFLIP$_L$ or NT control. (K) Extent of cell death, and cFLIP$_L$ protein levels in indicated BMDMs knocked down for cFLIP$_L$ using siRNA. (L) cFLIP$_L$ protein levels in B6 BMDMs knocked down for cFLIP$_L$ or NT control. Data presented are representative of three or more independent experiments. Cell death data are presented as the mean ± SD of triplicate wells.

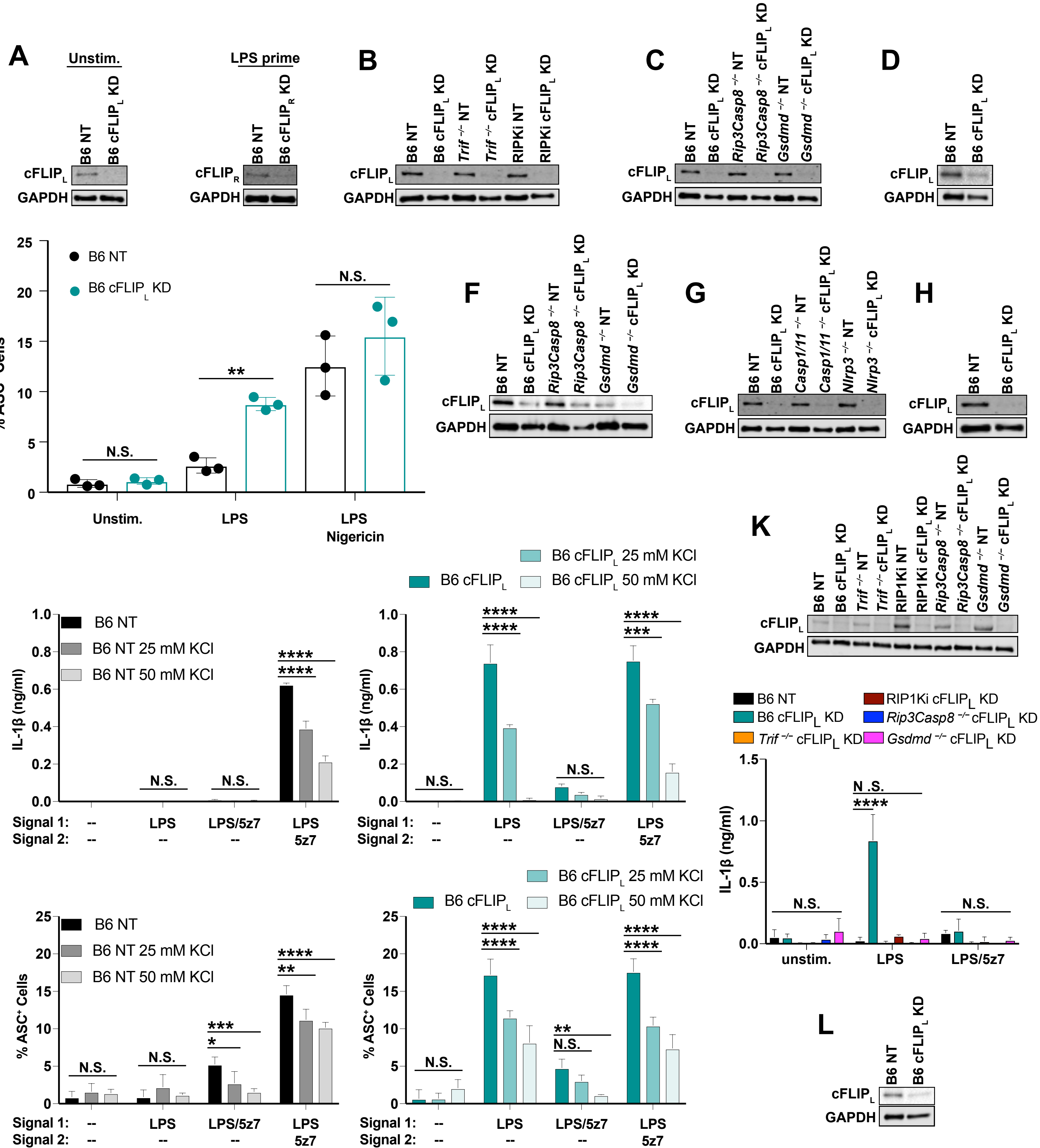

Fig. S4. (A-D) $cFLIP_L$ protein levels in indicated BMDMs knocked down for $cFLIP_L$ or transduced with a non-targeting control (NT). (E) Percentage of ASC^+ B6 BMDMs knocked down for $cFLIP_L$ after 2 hours of indicated treatment (extent of $cFLIP_L$ knockdown shown in panel D). (F-H) $cFLIP_L$ protein levels in indicated BMDMs knocked down for $cFLIP_L$ or NT control. (I, J) IL-1β release (I) and percentage of ASC^+ B6 BMDMs (J) knocked down for $cFLIP_L$ or NT control after 6 or 2 hours respectively of indicated treatment ± KCl (extent of $cFLIP_L$ knockdown shown in panel H). (K) Extent of IL-1β release, and $cFLIP_L$ protein levels in indicated BMDMs knocked down for $cFLIP_L$ using siRNA. (L) $cFLIP_L$ protein levels in B6 BMDMs knocked down for $cFLIP_L$ or NT control. Immunoblots and cell death data are representative of three or more independent experiments. IL-1β release and ASC percentage data are presented as the mean ± SD for triplicate wells from three or more independent experiments. Analysis of covariance (ANOVA) was used for comparison between groups: ns, nonsignificant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.